POLYMERIC ELECTROSPUN SCAFFOLDS: NEUREGULIN ENCAPSULATION AND BIOCOMPATIBILITY STUDIES IN A MODEL OF MYOCARDIAL ISCHEMIA

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ABSTRACT

Cardiovascular disease represents one of the major health challenges in modern times and is the number one cause of death globally. Thus, numerous studies are under way to identify effective cell- and/or growth factor-based therapies for repairing damaged cardiac tissue. In this regard, improving the engraftment or survival of regenerative cells and prolonging growth factor exposure have become fundamental goals in advancing these therapeutic approaches. Biomaterials have emerged as innovative scaffolds for the delivery of both cells and proteins in tissue engineering applications. In the present study, electrospinning was used to generate smooth homogenous polymeric fibers, which consisted of a PLGA/NCO-sP(EO-stat-PO) polymer blend encapsulating the cardioactive growth factor, Neuregulin-1 (Nrg). We evaluated the biocompatibility and degradation of this Nrg-containing biomaterial in a rat model of myocardial ischemia. Histological analysis revealed the presence of an initial acute inflammatory response after implantation, which was followed by a chronic inflammatory phase, characterized by the presence of giant cells. Notably, the scaffold remained in the heart after 3 months. Furthermore, an increase in the M2:M1 macrophage ratio following implantation suggested the induction of constructive tissue remodeling. Taken together, the combination of Nrg-encapsulating scaffolds with cells capable of inducing cardiac regeneration could represent an ambitious and promising therapeutic strategy for repairing diseased or damaged myocardial tissue.

Key words: Neuregulin, scaffold, tissue engineering, biocompatibility, cardiovascular disease

1. INTRODUCTION

The principal goal of tissue engineering (TE) is to replace or repair tissues that are damaged or worn out as a result of congenital abnormalities, disease, or injury through the use of sophisticated matrices, which enable efficient delivery of regenerative growth factors (GFs) and/or cells [1]. Notably, optimal scaffolds (SCs) used in TE approaches should promote cell attachment or migration as well as retention of implanted cells and/or GFs, while at the same time allowing for the diffusion of vital nutrients and cell products to stimulate efficient tissue repair and regeneration [2]. In this regard, the identification of innovative biomaterials is considered to be fundamental for advancing future TE applications.

Recently, TE has emerged as a promising therapeutic approach for restoring damaged heart tissue [3, 4], which is a major area of interest in regenerative medicine due to the high rates of cardiovascular disease (CVD)-related morbidity and mortality in modern times [5, 6]. Indeed, during myocardial infarction, occlusion of the coronary artery (or one of its smaller branches) causes ischemia and cardiac cell death. Although this damaged tissue is removed by macrophages, it is subsequently replaced by a non-functional fibrotic scar over a period of weeks through the activity of fibroblasts and endothelial cells [7]. Ultimately, this cardiac remodeling can produce cardiac insufficiency, resulting in a high risk of heart failure [8]. In this context, the prospect of engineering a device that effectively incorporates cells capable of regenerating heart tissue along with GFs, which can promote cell recruitment, vasculogenesis, and/or cardiomyocyte replication, is very attractive [9, 10].

Considering known cardioactive GFs, Neuregulin-1 (Nrg) has shown promise in preclinical studies in regenerative medicine and its therapeutic benefit in chronic heart disease is currently being investigated in several clinical trials (Clinicaltrials.gov identifier NCT01131637, NCT01214096, NCT01251406, NCT01258387, NCT01541202). Nrg is a member of the ErbB

receptor tyrosine kinase family and is indispensable for the development, structural

maintenance, and functional integrity of the heart [11]. We have previously demonstrated that

delivering polymeric microparticles encapsulating Nrg in a rat model of myocardial ischemia

(MI) led to improved cardiac function [12]. In fact, these microparticles decreased infarct size

and fibrosis, efficiently promoted vasculogenesis, cardiomyocyte proliferation, and the

recruitment of cardiac progenitors. Consequently, researchers have employed several

techniques to incorporate proteins, such as GFs, into fibrous biocompatible and biodegradable

SCs that allow cell attachment. In this regard, electrospinning (ES) has gained popularity as an

innovative method for generating SCs with ultrathin fiber diameters and large surface–volume

ratios, favoring the delivery of bioactive molecules [13, 14]. Specifically, ES is a manufacturing

process that involves applying an electrical charge to a liquid to draw out thin fibers (see Fig.

1). Parameters of the process can be controlled in order to optimize several fiber features such

as electric potential, flow rate, distance between the capillary and collection screen, motion,

size of target screen [collector] and needle gauge [15].

We have recently described the use of NCO-sP(EO-stat-PO) as an additive to PLGA for

construction of a fibrous SC using ES technology [16]. Notably, the amphiphilicity of this

molecule with 20% propylene oxide and 80% ethylene oxide enables anchorage of the

polymers in the PLGA matrix but also results in a hydrophilic surface of the fibers, leading to

reduced protein adsorption [17]. Nevertheless, the efficient incorporation of proteins into

electrospun fibers of this kind remains to be demonstrated. Moreover, the safety and efficacy

of these biomaterials for cardiovascular TE applications must be thoroughly assessed. In the

present study, we have designed, constructed, and characterized electrospun fibrous SCs,

which were incorporated with Nrg to facilitate heart tissue regeneration. Moreover, we

evaluated the biocompatibility of Nrg-containing SCs with damaged cardiac tissue using an *in

vivo* model of MI. With this proof-of-concept study we aimed to lay the foundations for the

biocompatibility and degradation of these protein encapsulating systems in order to perform the pertinent efficacy studies during the next stage. The results set out here will help us to design future *in vivo* studies.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals were provided by Sigma-Aldrich (Barcelona, Spain) unless specified otherwise in the text. Organic solvents were obtained from Panreac Quimica S. A. (Barcelona, Spain) and polymers were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Recombinant human Nrg (beta isoform) was provided by EuroBio-138 Sciences (Friesoythe, Germany). All western blot reagents were from Bio-Rad (Hercules, California, USA). Goat anti-human NRG-1 antibody (sc-1793) and horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Monoclonal rabbit anti-CCR7 (2059-1) was from Epitomics Inc. (Toronto, Ontario, Canada), and mouse anti-CD163 (MCA342R) was from AbD Serotec (Kidlington, UK). Isoflurane (IsoFlo®) used for anesthesia was purchased from Abbot Laboratories (Abbott Park, Illinois, USA), and sutures were from Ethicon (Somerville, New Jersey, USA).

2.2 Electrospinning conditions

Microfibers were produced at room temperature (RT; 20°C). A high voltage power supply was used to apply a 10-kV electrostatic field. The collector consisted of a rotating drum (6,000 rpm; length: 150 mm; diameter: 75 mm), which was placed at a distance of 140 mm from the needle tip (19-gauge, blunt-end). The polymer solution was electrospun at a rate of 1 mL/h, as controlled by a syringe pump (World Precision Instruments Inc.; Sarasota, Florida, USA), with a 30-min collection time. The resulting fibrous SC was cut and removed from the collector as a rectangular film (140 mm x 23.5 mm).

2.3 Fiber composition

We studied the following polymers: poly(lactic-co-glycolic acid) (PLGA); Resomer® 504, poly [(d,l-lactide-co-glycolide)-co-PEG] diblock Resomer® RGP d 50105 (PEG-PLGA); and PLGA/NCO-sP(EO-stat-PO). Prior to ES, the fibers were dissolved in acetone by stirring (450 µL; 5 min). For protein encapsulation, Nrg was first incorporated into an aqueous solution including polyethyleneglycol 200 (PEG200), and tri-fluoro-acetic-acid (TFA) was subsequently added. This aqueous phase was then included into the organic phase by stirring until homogenization (1 min) to obtain the emulsion for ES. The formulation components were evaluated during fiber preparation in order to obtain smooth fibers which showed adequate cytokine encapsulation and were suitable for cell adhesion. A 50-µm pore size was considered to be appropriate for proper vascularization to supply oxygen and nutrients to SC-associated cells [10]. The following additional variables were evaluated: polymer composition, amount of PEG200 in the aqueous solution, composition of the aqueous solution, protein loading in the fibers, and differential TFA concentrations. All fibers were collected as a film on the surface of a metallic rotating drum.

2.4 Scanning electron microscopy imaging

Small pieces of SC (0.4 mm x 0.4 mm) were prepared for imaging using a sputter coater (Emitek K550), and surface morphology was visualized with a scanning electron microscope (Zeiss DSM 940A).

2.5 Nrg encapsulation efficiency

Protein loading in the fibers was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot, as described elsewhere [12]. Briefly, fibers (2 mg) were dissolved in dichloromethane (40 µL) and electrophoresed (200 V, 50 min).

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Separated proteins were then transferred to nitrocellulose membranes (350 mA, 1 h, RT), which were incubated with anti-Nrg (1:50, 4°C, overnight) followed by HRP-conjugated donkey anti-goat IgG (1:2,000, RT, 2 h). LumiLight Plus Western Blot substrate was used to detect chemiluminescence (Roche, Mannheim, Germany), and an ImageQuant RT ECL Imager with ImageQuant TL Software was employed for analysis of protein bands (GE Healthcare, Fairfield, Connecticut, USA). An Nrg standard curve was prepared and analyzed for quantification.

2.6 *In vivo* animal model and scaffold adhesion

All experiments involving animals were performed according the "Principles of Laboratory Animal Care" from the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" [18]. Moreover, procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals. A previously described rat model of cardiac ischemia was used for our *in vivo* studies [19]. For this, female Sprague Dawley rats (250–300 g) were anesthetized (4% isoflurane) in an induction chamber and subsequently intubated with ventilation maintained at 80–90 cycles/min under continuous anesthesia (isoflurane 2%). Prior to surgery, fentanyl (0.15 mg/kg) was administered intraperitoneally. A left thoracotomy was performed through the fourth intercostal space, and the left anterior descending coronary artery was ligated using a non-resorbable suture (PROLENE 7.0; 2–3 mm from its origin). The chest was then closed in layers, and following 1 week, it was reopened in order to apply SC (1.2 cm x 1.2 cm) over the infarcted area. After surgery, ketoprofen (5 mg/kg) was subcutaneously administered for 3 days, and antibiotic (enrofloxacin; Alsir lechones, Esteve Veterinaria; 25 mg/kg) was supplied in the drinking water for 7 days. Animals were sacrificed at 24 h, 1 week, 1 month, and 3 months (two animals per group). Prior to sacrifice, they were anesthetized and perfusion-fixed. Hearts were harvested, fixed in 4% formaldehyde overnight (4°C), and embedded in paraffin for histological studies.

2.7 Histological analysis

Hearts were cut into three pieces (apical, mid-ventricular, and basal) and embedded in paraffin for sectioning (5 μm). Haematoxylin-eosin staining was performed by submersion in Harris haematoxylin followed by treatment with 37% HCl and Li_2CO_3 solution for differentiation. Samples were subsequently immersed in 0.5% eosin, dehydrated, and mounted in DPX. Immunolabeling was employed to determine macrophage phenotype. Tissue slides were deparaffined and antigen retrieval was performed. For analyzing the M1 phenotype, the microwave–citrate method was utilized (citrate 10 mM, pH 6; microwave heating for 20 min), whereas for the M2 phenotype, trypsin antigen retrieval was used (trypsin 0.2% in calcium chloride 0.1%, 5 min, 37°C). After washing, sections were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline and then incubated overnight (4°C) with primary antibodies (anti-CCR7 for M1 and anti-CD163 for M2) diluted 1:100 in blocking solution. Samples were washed and incubated with corresponding secondary antibodies (anti-rabbit FITC and anti-mouse Alexa Fluor-594) for 30 min at RT, and DAPI was used to stain nuclei. M1 and M2 macrophages were visualized and quantified using a Zeiss Axioplan 2ie microscope equipped with epifluorescence optics. Six images per section were analyzed (8 sections per animal, 40X magnification), and digital images were processed using ImageJ software.

2.8 Statistical analysis

Data are expressed as mean ± SEM. To analyze statistical significance for group comparisons, the student's t-test was used. *P*-values correspond to two-tailed unpaired *t*-tests, and $p < 0.05$ was considered to be statistically significant. All analyses were conducted using Prism software (GraphPadsoftware, San Diego, CA, USA).

3   RESULTS

3.1 Fiber preparation

In order to engineer effective SCs for cardioregenerative applications, we first aimed to optimize our ES procedure by testing various fiber compositions (Table 1). For this, we prepared fibers containing PLGA, PEG-PLGA, and a combination of both polymers (1:1 ratio). Notably, addition of increasing amounts of NCO-sP(EO-stat-PO) to the polymer mixture led to a dose-dependent alteration in fiber structure and integrity, with higher amounts of NCO-sP(EO-stat-PO) leading to more heterogeneous and fused fibers (Fig. 2A, compare left panel to far right panel). Therefore, to overcome this limitation, TFA was added to improve conductivity. Although lower concentrations of TFA diminished structural changes in the fibers, higher concentrations of TFA resulted in fibers that were less uniform and showed a tendency for fusion (Fig. 2B, compare left panel to far right panel). Finally, PEG200 was added to the aqueous solution in order to protect added proteins against degradation by organic solvents and to prevent hydrophobic protein–polymer interactions. In this regard, homogeneous fiber formation required the addition of a minimum of 30 μL of PEG200. In contrast, although 20 μL of PEG200 allowed fibers to be electrospun, the resulting products fused in the collector (Fig. 2C, left panel). Fibers could not be formed in the presence of lower PEG200 concentrations.

3.2 The effects of proteins and salts on ES fiber formation

After optimizing the conditions for fiber preparation, we next wanted to analyze the impact of incorporating proteins during the ES process. Indeed, efficient fiber production in the presence of proteins would be a prerequisite for encapsulating GFs into our electrospun SCs. Proteins are labile molecules that are typically dissolved in buffered solutions containing salts and carrier proteins to protect them against degradation, and this characteristic had to be considered when designing our protein-integrated SCs for TE applications. In the context of fiber formation, we found that high amounts of BSA carrier protein led to increasing heterogeneity of fiber morphologies and diameter (Fig. 3). Moreover, we found that including salts in the protein solution negatively affected fiber structure (Fig. 4). Thus, these findings

demonstrated that in order to prepare structurally intact protein-containing fibers, the protein solutions blended with the polymers must be devoid of carrier proteins and salts.

3.3 Encapsulation efficiency

The above findings suggested that Nrg might be effectively encapsulated within ES fibers under defined conditions. Therefore, we next tested the efficiency of Nrg incorporation into fibers using six different SC compositions (Table 2). Following production of fibers with these distinct conditions, we evaluated the level of Nrg loading using western blot analysis. For this, the amount of protein retained was compared with the protein initially included in the emulsion and expressed as a percentage (see Fig. 5 as example). These results indicated that all six SC conditions were suitable for protein encapsulation, with encapsulation efficiency (EE) values found to be over 85% in all cases.

3.4 *In vivo* studies and histological analysis

Based on our initial characterization of the Nrg-containing SCs, we chose to use PLGA and PLGA/NCO-sP(EO-stat-PO) polymer with a low TFA concentration (composition 6 in Table 1) for subsequent *in vivo* biocompatibility studies. We first assessed the interaction of our SCs with injured cardiac tissue over a period of 3 months following implantation in a rat model of ischemia (Fig. 6A-D). Histological analyses revealed that the SCs efficiently adhered to and integrated in the cardiac tissue following implantation, remaining for up to 3 months (Fig. 6). Also, we noted an inflammatory infiltrate that appeared in the area of the implant, with cells penetrating and diffusing through the adhered fibrous SC (Fig. 6, black double arrows). Specifically, acute inflammation was observed at 24 h and 1 week after implantation (Fig. 6 A and B, respectively). This was followed by a phase of chronic inflammation, which was observed at 1 month and persisted until 3 months post implantation (Fig. 6 C and D, respectively). Notably, at the later time points, fibrotic tissue could be seen adjacent to the

biomaterial (Fig. 6 C and D, blue arrow), with the inflammatory infiltrate in the SC visibly reduced compared to the first week. Additionally, Langham giant cells, displaying a characteristic horseshoe nuclei arrangement, could be observed near the SCs at 1 and 3 months post implantation (Fig. 6E-H, black arrows).

3.5 M2:M1 macrophage phenotype

We next aimed to analyze whether our Nrg-containing SC exerted itself a positive or a negative impact on cardiac tissue remodeling when implanted into ischemic rats. In order to assess the regenerative effects of our biomaterial, we examined the M2:M1 macrophage ratio at 24 h, 1 week, and 1 month after implantation. Indeed, M1 and M2 macrophages were recently shown to play distinct roles in tissue remodeling following injury [20, 21]. Thus, we employed immunofluorescence imaging to analyze the number of CCR7- and CD163-expressing cells in the injured cardiac tissue over time (M1 and M2 phenotypes, respectively). Notably, we found that the M2:M1 ratio significantly increased up to 1 month after implantation (Fig. 7), suggesting that the Nrg-containing fibers themselves have a positive effect on cardiac remodeling.

4   DISCUSSION

In the present study, ES was successfully employed to generate Nrg-encapsulating fibers from a mixture of PLGA/NCO-sP(EO-stat-PO) polymers. Following implantation of these fibrous SCs into a rat model of myocardial ischemia, histological analyses revealed that the biomaterial adhered to and was integrated into the cardiac tissue. In addition, we observed the presence of an initial acute inflammatory response to the SC, which was followed by a chronic inflammatory phase. Furthermore, increase in the M2:M1 macrophage ratio indicated constructive remodeling of injured cardiac tissue following SC implantation.

In order to prepare the electrospun fibers for our scaffolds we tested several parameters. We also included TFA to increase the electrical conductivity of the polymer solution. Indeed, it is necessary for the emulsion to reach a critical charge to allow the fluid jet to erupt from the droplet at the tip of the needle, creating a Taylor cone (see Fig. 1). The polymer mixture can then move toward the grounded collector, which presents a lower potential for fiber formation [22]. Indeed, TFA led to improved fiber morphology in our experiments. However, it must also be noted that excess TFA in the emulsion produced negative effects, including fiber fusion. In addition, regarding PEG200, fiber formation did not occur when the amount of PEG200 was too low, which likely resulted from high viscosity. In this respect, a minimum viscosity may be required to prevent breakage of the polymer jet as it travels from the injector to the collector. On the other hand, if the viscosity is too high, then the solution can dry on the needle tip and prevent ES from occurring.

Our analysis indicated that Nrg could be efficiently incorporated into the electrospun fibers. In fact, our GF encapsulation rate (85%) was much higher than that obtained by other groups' engineering electrospun PLGA fibers. For example, Sahoo *et al*. prepared PLGA nanofibers containing basic fibroblast growth factor (bFGF) with 54% EE [23]. Moreover, Nie *et al.* evaluated incorporation of bone morphogenetic protein-2 (BMP-2) into several fiber compositions, observing EE values from 44 to 66% [24]. Our findings demonstrate that high levels of bioactive proteins can be efficiently incorporated into electrospun fibers for use in TE applications.

For the animal studies, we chose to employ PLGA/NCO-sP(EO-stat-PO) with a low quantity of TFA for fiber formation, as this composition appeared to be the most suitable for cell adhesion and *in vivo* study. Additionally, our Nrg-containing SCs were directly applied on the injured cardiac tissue without the use of sutures or natural glues, effectively adhering and integrating

with the tissue. In contrast to this, previously described biomaterials proved unable to integrate into the heart tissue due to issues with rigidity [25]. Therefore, the fact that our polymeric SCs were easily attached and adapted to the cardiac tissue represents an important advantage that can facilitate effective cardioregeneration. Furthermore, our flexible biomaterials were elastic enough to resist the stress and strain of heart muscle contractions, remaining in one piece throughout our experiments.

With regard to the local immune reaction, we initially observed an acute inflammatory response, which was followed by chronic inflammation involving macrophages and giant cells. When macrophages are unable to phagocytose the biomaterial, they fuse to form multinucleated giant cells, which adhere to the surface of the biomaterial and release mediators of degradation (e.g., reactive oxygen species, degradative enzymes, and acids) [26]. Secretion of these factors over a short period of time can lead to device failure, limiting the beneficial effect of regenerative SCs. In fact, this reaction commonly occurs during the healing process after MI or following biomaterial implantation [27, 28]. Thus, since our SCs demonstrated long-term structural integrity in the cardiac tissue, it may indicate that the immunological response was not damaging to the biomaterials and that prolonged cytokine release occurred. Nevertheless, long-term studies will be necessary to determine *in vivo* degradation rates as well as the duration of active Ngr release from our fibrous SCs.

Recently, macrophage polarization toward the M1 or M2 phenotype was found to play a role in constructive tissue remodeling [20, 21]. The M1 phenotype corresponds to classically-activated proinflammatory macrophages, whereas M2 macrophages are associated with regulatory and homeostatic functions (e.g., tissue regeneration) [29]. Thus, switching from the M1 to the M2 phenotype can promote progenitor cell differentiation and tissue remodeling [20, 30]. For this reason, increase in the M2:M1 ratio following implantation of our Nrg-

containing SCs might suggest that the initial immune reaction to the implant evolved into a more constructive response over time. This would correspond to our observation that PLGA/NCO-sP(EO-stat-PO) ES fibers induce M2 phenotype macrophages in vitro [31]. However, more long-term studies will be needed to analyze the full regenerative potential of this innovative biomaterial in vivo.

5   CONCLUSIONS

Taken together, we demonstrated that Nrg-containing fibers engineered via ES technology using a mixture of PLGA/NCO-sP(EO-stat-PO) polymers displayed efficient adherence, integration, and biocompatibility with injured cardiac tissue. Moreover, SCs generated from these fibers yielded results consistent with constructive tissue remodeling. These findings suggest that combining Nrg-encapsulating SCs with cells capable of inducing cardiac regeneration might represent an ambitious and promising therapeutic strategy for repairing damaged or diseased myocardial tissue in humans. This proof-of-concept study establishes the foundation for future *in vivo* efficacy studies using these Nrg-containing fibers.

ACKNOWLEDGEMENTS

REFERENCES

[1] Simón-Yarza, T., Garbayo, E., Tamayo, E., Prósper, F., Blanco-Prieto, M.J. Drug delivery in tissue engineering: general concepts. In: Alonso, M.J., and Csaba, N.S., eds. Nanostructured biomaterials for overcoming biological barriers. Cambridge: RSC Publishing, 2012, pp. 501-525.

[2] Garg, T., Singh, O., Arora, S., Murthy, R. Scaffold: a novel carrier for cell and drug delivery. Crit Rev Ther Drug Carrier Syst 29, 1, 2012.

[3] Doppler, S.A., Deutsch, M.A., Lange, R., Krane, M. Cardiac regeneration: current therapies-future concepts. J Thorac Dis 5, 683, 2013.

[4] Lundberg, M.S. Cardiovascular tissue engineering research support at the National Heart, Lung, and Blood Institute. Circ Res 112, 1097, 2013.

[5] World Heart Organization, World health statistics 2012. http://www.who.int/gho/publications/world_health_statistics/2012/en/index.html. Accessed: September 2014.

[6] World Health Organization (WHO). Cardiovascular diseases (CVDs) fact sheet no. 317. http://www.who.int/mediacentre/factsheets/fs317/en/. Updated March 2013. Accessed: September 2014.

[7] Martin, K., Huang, C.L., Caplice, N.M. Regenerative approaches to post-myocardial infarction heart failure. Curr Pharm Des 20, 1930, 2014.

[8] Kurrelmeyer, K., Kalra, D., Bozkurt, B., Wang, F., Dibbs, Z., Seta, Y., et al. Cardiac remodeling as a consequence and cause of progressive heart failure. Clin Cardiol 21, I14, 1998.

[9] Lakshmanan, R., Krishnan, U.M., Sethuraman, S. Living cardiac patch: the elixir for cardiac regeneration. Expert Opin Biol Ther 12, 1623, 2012.

[10] Venugopal, J.R., Prabhakaran, M.P., Mukherjee, S., Ravichandran, R., Dan, K., Ramakrishna, S. Biomaterial strategies for alleviation of myocardial infarction. J R Soc Interface 9, 1, 2012.

[11] Mendes-Ferreira, P., De Keulenaer, G.W., Leite-Moreira, A.F., Bras-Silva, C. Therapeutic potential of neuregulin-1 in cardiovascular disease. Drug Discov Today 18, 836, 2013.

[12] Formiga, F.R., Pelacho, B., Garbayo, E., Imbuluzqueta, I., Diaz-Herraez, P., Abizanda, G., et al. Controlled delivery of fibroblast growth factor-1 and neuregulin-1 from biodegradable microparticles promotes cardiac repair in a rat myocardial infarction model through activation of endogenous regeneration. J Control Release 173, 132, 2014.

[13] Ji, W., Sun, Y., Yang, F., van den Beucken, J.J., Fan, M., Chen, Z., et al. Bioactive electrospun scaffolds delivering growth factors and genes for tissue engineering applications. Pharm Res 28, 1259, 2011.

[14] Braghirolli, D.I., Steffens, D., Pranke, P. Electrospinning for regenerative medicine: a review of the main topics. Drug Discov Today 19, 743, 2014.

[15] Rim, N.G., Shin, C.S., Shin, H. Current approaches to electrospun nanofibers for tissue engineering. Biomed Mater 8, 2013.

[16] Grafahrend, D., Heffels, K.H., Beer, M.V., Gasteier, P., Moller, M., Boehm, G., et al. Degradable polyester scaffolds with controlled surface chemistry combining minimal protein adsorption with specific bioactivation. Nat Mater 10, 67, 2011.

[17] Heyes, C.D., Groll, J., Moller, M., Nienhaus, G.U. Synthesis, patterning and applications of star-shaped poly(ethylene glycol) biofunctionalized surfaces. Mol Biosyst 3, 419, 2007.

[18] National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals, 8th edition. Washington D.C.: National Academies Press 2011.

[19] Simon-Yarza, T., Tamayo, E., Benavides, C., Lana, H., Formiga, F.R., Grama, C.N., et al. Functional benefits of PLGA particulates carrying VEGF and CoQ10 in an animal of myocardial ischemia. Int J Pharm 454, 784, 2013.

[20] Brown, B.N., Londono, R., Tottey, S., Zhang, L., Kukla, K.A., Wolf, M.T., et al. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. Acta Biomater 8, 978, 2012.

[21] Badylak, S.F., Valentin, J.E., Ravindra, A.K., McCabe, G.P., Stewart-Akers, A.M. Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng Part A 14, 1835, 2008.

[22] Ramakrishna, S., Fujihara, K., Wee-Eong, T. An introduction to electrospinning and nanofibers. Singapore: World Scientific Publishing, 2005.

[23] Sahoo, S., Ang, L.T., Goh, J.C., Toh, S.L. Growth factor delivery through electrospun nanofibers in scaffolds for tissue engineering applications. J Biomed Mater Res A 93, 1539, 2010.

[24] Nie, H., Soh, B.W., Fu, Y.C., Wang, C.H. Three-dimensional fibrous PLGA/HAp composite scaffold for BMP-2 delivery. Biotechnol Bioeng 99, 223, 2008.

[25] Araña, M., Pena, E., Abizanda, G., Cilla, M., Ochoa, I., Gavira, J.J., et al. Preparation and characterization of collagen-based ADSC-carrier sheets for cardiovascular application. Acta Biomater 9, 6075, 2013.

[26] Kao, W.J. Evaluation of protein-modulated macrophage behavior on biomaterials: designing biomimetic materials for cellular engineering. Biomaterials 20, 2213, 1999.

[27] Anderson, J.M., Rodriguez, A., Chang, D.T. Foreign body reaction to biomaterials. Semin Immunol 20, 86, 2008.

[28] Freytes, D.O., Santambrogio, L., Vunjak-Novakovic, G. Optimizing dynamic interactions between a cardiac patch and inflammatory host cells. Cells Tissues Organs 195, 171, 2012.

[29] Badylak, S.F., Gilbert, T.W. Immune response to biologic scaffold materials. Semin Immunol 20, 109, 2008.

[30] Keane, T.J., Londono, R., Turner, N.J., Badylak, S.F. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials 33, 1771, 2012.

[31] Bartneck, M., Heffels, K.H., Pan, Y., Bovi, M., Zwadlo-Klarwasser, G., Groll, J. Inducing healing-like human primary macrophage phenotypes by 3D hydrogel coated nanofibres. Biomaterials 33, 4136, 2012.

FIGURE LEGENDS

Figure 1. Electrospinning process: main components of the system and Taylor cone formation. A voltage is applied to the polymer solution that becomes charged promoting its eruption from the needle towards the grounded collector (this point of eruption receives the name of Taylor cone). The jet dries and fibers are drawn on the collector surface forming the scaffold.

Figure 2. Optimization of fiber composition. Fibers were prepared with: (A) different amounts of sP(EO-stat-PO) (left to right: 5 mg, 10 mg, 15 mg, and 20 mg/150 mg total polymer; scale bars: 10 μm); (B) different TFA concentrations (left to right: 0, 0.5, 2, and 10 μL/mL; scale bars: 10 μm); (C) different amounts of PEG200 (left to right: 20, 30, 40, and 50 μL/150 total polymer; scale bars: 5 μm).

Figure 3. Effects of carrier protein on fiber formation. Fibers were prepared in the presence of various amounts of BSA (left to right: 10, 25, 50, 100, and 150 μg/150 total polymer; scale bars: 20 μm).

Figure 4. Effects of salt on fiber formation. Fibers were prepared in presence of water (left) or PBS (right) in the aqueous solution. Scale bars: 5 μm.

Figure 5. Encapsulation efficiency. Western blot bands corresponding to Nrg standard curve and Nrg encapsulated in the scaffold (in duplicate).

Figure 6. Biocompatibility of the non-loaded fibers. SCs in the tissue (double arrow) and fibrotic tissue (blue arrow) after 24 h (A), 1 week (B), 1 month (C), and 3 months (D). Scale bars: 50 μm. (Haematoxylin-eosin staining; 10X magnification). Images E-H show the presence of Langham giant cells near the implanted SC. Implantation site after 24 h (E), 1 week (F), 1 month (G), and 3 months (H). Arrows indicate the presence of foreign body giant cells. Scale bars: 20 μm. (Haematoxylin-eosin staining; 40X magnification).

Figure 7. Macrophage polarization. Immunofluorescent images showing examples of the host macrophage response to scaffolds after 1 month. Scale bars: 10 μm. CCR7 (M1) = red, CD163

(M2) = green, and DAPI (nuclei) = blue. The graph on the right shows the ratio of M2:M1 expressing cells (indicated as CD163+/CCR7+) at 24 h, 1 week, and 1 month after implantation (**p<0.001).

TABLE LEGENDS

Table 1. Fiber composition parameters

Legend: PLGA, poly(lactic-co-glycolic acid); PEG, polyethyleneglycol; TFA, tri-fluoro-acetic-acid;

PBS, phosphate-buffered saline; BSA, bovine serum albumin.

Table 2. Various compositions of Nrg-containing scaffolds

Legend: PLGA, poly(lactic-co-glycolic acid); PEG, polyethyleneglycol; TFA, tri-fluoro-acetic-acid;

Nrg, Neuregulin-1.